Post-Natal Stem Cells for Dental and Craniofacial Repair

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Summary: The post-natal bone marrow contains a subset of stromal cells (skeletal stem cells) that have the ability to form bone, cartilage, hematopoietic stroma, adipocytes and perhaps other tissues as determined by clonal analysis and *in vivo* transplantation into immunocompromised mice. Similar, but not identical, cells have also been isolated from peripheral blood, although they are rare in humans. Dental pulp of permanent and deciduous teeth, and periodontal ligament also contain stem cells that have the ability to regenerate a dentin/pulp-like complex, and cementum and periodontal ligament-like structures, respectively. Using appropriate *ex vivo* expansion conditions and scaffolds, animal models have been created to demonstrate the efficacy of *ex vivo* expanded populations that contain skeletal stem cells to regenerate a number of tissues. With these techniques in hand, it is possible to consider the recreation of a viable tooth and supporting structures for restoration of normal masticatory function.

Key words: tissue engineering, oral hard tissues, post-natal stem cells

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INTRODUCTION

There is certainly no single scientific topic that has more keenly attracted the attention of not only scientific and clinical investigators, but that of the public at large, as that of stem cells and their potential use in regenerative medicine. A current read of the literature suggests that virtually every tissue in the body contains a cell that has some sort of regenerative capacity. However, it is not clear that all of these cells fully qualify as stem cells based on current definitions. By strict definition, a single stem cell has the ability to completely reconstitute an entire tissue in the body, and that it has the ability to self-renew and maintain itself for the lifetime of the organism, best demonstrated by *in vivo* transplantation. Unfortunately, with the notable exception of the hematopoietic stem cell (HSC) and the hematopoietic system, such *in vivo* assays are either not routinely employed, or currently available in many tissue systems. Further assumptions based on *in vitro* analyses ascribe clonogenicity, extensive proliferative capacity, and an undifferentiated phenotype as properties of a stem cell. But there are notable exceptions to these assumptions, the ultimate of which is demonstrated by nuclear reprogramming, whereby a nucleus derived from a mature, post-natal cell can regenerate an entire organism when placed in enucleated oocyte cytoplasm.

In mapping the course of embryonic development, the fertilized egg is the epitome of a toti-potent stem cell, able to give rise independently to all of the tissues of the body. Through a tightly controlled series of divisions, it gives rise to the blastocyst, composed of an outer layer of cells that form trophoblasts and placental membranes, and the inner cell mass. While the inner cell mass is fleetingly toti-potent, it rapidly gives rise to the three embryonic germ layers (ectoderm, mesoderm, endoderm) that contain fetal stem cells with more restricted lineage multi-potentiality. Embryonic stem cells, derived from the inner cell mass and expanded *ex vivo*, have the ability to contribute to formation of every tissue in the body, but they cannot, by themselves, reform an entire organism, and thus are thought of as pluri-potential (for reviews see Lanza, 2004a,b).

POST-NATAL STEM CELLS

Based on the astute observations of histologists and hematologists working in the late 1800s and early 1900s, it was hypothesized that post-natal stem cells must exist, in order to replenish tissues that are rapidly turned over, such as blood, skin and the gastro-intestinal tract (reviewed in Robey, 2000). Definitive identification of a hematopoietic stem cell in the 1960s supported this hypothesis, and subsequently post-natal stem

cells were identified in tissues with much slower rates of replacement and the ability to completely repair (such as bone), in tissues with limited ability to repair (such as muscle), and in tissues never thought to repair (such as nervous tissue). Confirmation of stem cells in other post-natal tissues is slowly emerging. Furthermore, it is also a possibility that current *ex vivo* manipulations create cell types that do not exist *in vivo*. For example, embryonic stem cells continuously proliferate and do not differentiate (without given instructions), characteristics that are not displayed by cells in the inner cell mass. Nonetheless, there are clear examples of how 'stem' cells are of interest, not only from a biological point of view, but also in terms of tissue engineering and regenerative medicine (Bianco et al, 2001b; Robey et al, 2004).

THE ADULT BONE MARROW ORGAN: A CENTRAL REPOSITORY OF STEM CELLS

The adult bone marrow organ is compromised of hematopoietic tissue, formed and supported by a highly interconnected stromal cell network, and nourished by a specialized vasculature that controls the egress and ingress of cells, to and from the circulation (Bianco et al, 1998). Bone marrow has long been known to be the home of the HSC and the skeletal stem cell (SSC), but based on the results of bone marrow transplantation, cells of donor origin also appear to become incorporated into many, if not all tissues, leading to the speculation that bone marrow contains stem cells for liver, muscle, blood vessels, etc. However, there is limited definitive proof that marrow cells of donor origin actually differentiate and function in recipient tissues, or whether they may be serving as 'helper' cells in some fashion. Regardless of the outcomes of future studies aimed at answering these questions, bone marrow emerges as an easily accessible, potentially central repository of post-natal stem cells of great utility (Robey et al, 2004).

POST-NATAL SKELETAL STEM CELLS

From the pioneering work of Friedenstein, and Owen and coworkers, it has long been know that *ex vivo* expanded bone marrow stromal cells contain a subpopulation of post-natal stem cells (Friedenstein et al, 1966; Owen, 1988). When a single cell suspension of marrow is plated at low density, a single colony forming unit-fibroblast (CFU-F) rapidly adheres and proliferates to form a colony (Fig 1). Clonal analysis indicates that between 10-20% of CFU-Fs are true SSCs, capable of completely regenerating a bone/marrow organ (composed of bone-forming osteoblasts, hematopoiesis-supportive stroma and associated adipocytes) upon *in vivo* transplantation with an appropriate carrier (hydroxyapatite/tricalcium phosphate, HA/TCP) (Fig 2a), and to form cartilage when cultured under anaerobic conditions in micro-mass cultures (Fig 2b), (four different cell phenotypes) (Krebsbach et al, 1997; Kuznetsov et al, 1997; Muraglia et al, 2003). Their multi-potentiality may also extend into other tissues associated with the skeleton such as tendons, ligaments and muscle, and perhaps beyond tissues of the skeleton, although definitive proof is lacking.

FLEXIBILITY AND PLASTICITY

While it was initially useful to compare the HSC system to the SSC system, significant differences have emerged, which highlight the need for being less dogmatic concerning the properties of post-natal stem cells. For example, the progeny of the hematopoietic system are short-lived and continuously formed, whereas the progeny of the skeletal system are formed at specific stages of development, and only slowly turnover after puberty. Blood is turned over very rapidly (ranging from 18 hours up to 120 days, depending on the cell type), whereas after puberty, some sites in the skeleton may be renewed only two to four times more. Consequently, there may be inherent differences in the capacity for extensive prolifer-

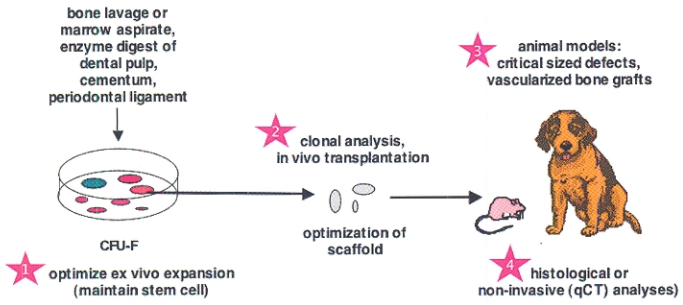

Fig 1 Scheme of isolation and characterization of post-natal SSCs from bone marrow, peripheral blood, dental pulp, cementum and periodontal ligament. Single cell suspensions are plated at low density in culture conditions that have been optimized to maintain a sub-population of stem cells. The CFU-Fs adhere and proliferate to form colonies. Colonies are isolated and cells are expanded in number, and attached to HA/TCP particles (to date, the most optimal scaffold) for transplantation into immunocompromised mice. In pre-clinical studies, animal models of critical sized defects and vascularized bone grafts were generated to determine efficacy of *ex vivo* expanded SSCs to regenerate bone by histological and qCT analysis.

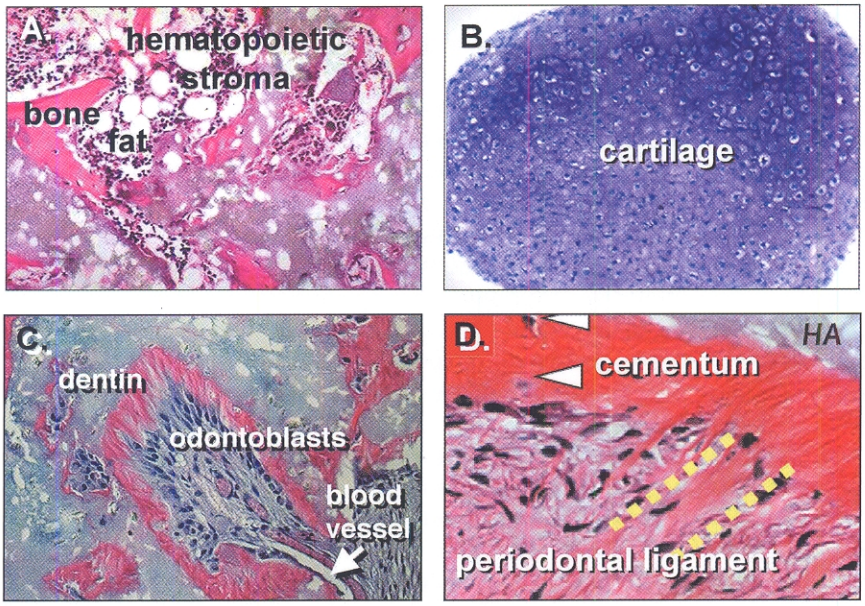

Fig 2 Histological appearance of cell populations transplanted with HA/TCP particles into immunocompromised mice. **(a)** Transplanted bone marrow stromal cells from bone, hematopoietic supportive stroma and adipocytes. **(b)** When cultured at high density in anaerobic conditions, bone marrow stromal cells form cartilage as demonstrated by staining with toluidine blue. **(c)** Dental pulp cells from third molars form primary dentin associated with odontoblasts and a pulp-like complex (modified from Gronthos et al, 2000). **(d)** Cells from periodontal ligament form cementum and a PDL-like structure, with associated Sharpey's fibers (delimited by yellow lines) (modified from Seo et al, 2004).

ation and self-renewal between the stem cells of the two systems. The hematopoietic system exists in a single-dimensional fluid phase, and is easily replaced by fluid exchange, whereas the skeletal system is a complicated three-dimensional solid phase. It is not clear how the skeletal system can easily be replaced other than by direct orthotopic application. But perhaps the biggest difference between the two systems relates to 'flexibility'. As the HSC begins to differentiate, it becomes more and more specified, such that conversion from one mature cell type to another is never observed. However, this is not the case in the skeletal cell system, where there are instances in disease states of mature osteoblasts to form fibrotic tissue (as in hyperparathyroidism), or to form more red marrow-supportive stroma (in cases of massive hemorrhage or hemolytic anemias), or to undergo formation of marrow adipocytes as is seen in aging and involutional osteoporosis. This 'flexibility' can also be demonstrated *in vitro* by looking at the differentiation pattern of individual colonies that can be first induced to form adipocytes, and subsequently be induced to form osteoblasts. While it has not been demonstrated that it is a single cell that first existed as a CFU-F, then as an adipocyte, and subsequently as an osteoblast exists, it is clear that the progeny of the original CFU-F had the ability to shift from one phenotype to another (Cherman and Robey, unpublished). The question remains whether or not a SSC was maintained within the milieu created by the changing the microenvironment. However, this type of flexibility has not been demonstrated in CFU's isolated from the hematopoietic system.

While the question of true 'flexibility' of SSCs is not yet definitive, the question of 'plasticity' of post-natal stem cells in general, is even less so. 'Plasticity' has been recently defined as the ability of a post-natal stem cell to differentiate into a cell type outside of the germ layer from which it arose, so called trans-germal differentiation. Using isolated cell populations and *in vivo* transplantation, there are a number of reports of trans-germal differentiation: neural stem cells (ectoderm) forming blood and muscle (mesoderm); HSCs (mesoderm) forming liver cells (endoderm) and nerve cells (ectoderm); SSCs (mesoderm) forming neuronal tissue (ectoderm). In some cases, it appears that the finding is a result of cell fusion of the donor cell with recipient cells, in others it appears that donor cells do incorporate into tissues, but it is not clear if they truly trans-differentiated or serve as some type of a support cell. Either way, the phenomena are worth pursuing within the context of tissue engineering and regenerative medicine (reviewed in Robey et al, 2004).

OTHER SOURCES OF SKELETAL STEM CELLS

Using the techniques that had been developed for the isolation and characterization of stem cells from bone marrow (clonogenic isolation and *in vivo* transplantation with an appropriate carrier), a number of other sources have been identified that contain stem cells that are able to regenerate skeletal and associated soft tissues. Although it had long been postulated that a SSC might exist in the circulation, isolation and characterization of a true, non-hematopoietic, SSC has remained

elusive. Peripheral blood has been found to give rise to adherent, clonogenic cells that are able to form bone upon *in vivo* transplantation, and to form adipocytes and cartilage *in vitro*. However, in humans, these cells are extremely rare, and the circumstances and purposes for their existence in the circulation are unknown (Kuznetsov et al, 2001). Although a recent study has identified a cell type (based on expression of a bone matrix protein, osteocalcin) that suggests that circulating SSCs are relatively abundant during puberty (Eghbali-Fatourechi et al, 2005), it is not yet clear if these cells are capable of creating bone de novo (a necessary property for classification), or inducing bone within the recipient organism.

Furthermore, these techniques have been applied to hard and soft tissues of dental and periodontal tissues. Dental pulp from either the permanent dentition (primarily third molars) or from deciduous teeth (primarily incisors) were isolated, treated with enzyme solutions to liberate a single cell suspension, and were found to give rise to clonal populations of cells that upon *in vivo* transplantation with HA/TCP, gave rise to a dentin/pulp-like complex (Fig 2c) (Gronthos et al, 2000; Miura et al, 2003). Interestingly, dental pulp cells from deciduous teeth (SHED, Stem cells from Human Exfoliated Deciduous teeth) also developed nerve-like character upon using culture conditions known to support neuronal stem cells, although further work is needed to determine their true potential for neuronal differentiation and function. In a similar fashion, shavings of cementum were found to give rise to clonogenic cells capable of regenerating a cementum-like structure (Grzesik et al, 1998), and cells isolated from the periodontal ligament formed both PDL and cementum like structures, upon transplantation (Fig 2d) (Seo et al, 2004).

MAJOR QUESTIONS: RELATEDNESS, ORIGIN AND DIFFERENTIATION CAPACITY OF POST-NATAL SKELETAL STEM CELLS

Taken together, these results have established that some type of a stem cell can be isolated from a variety of dental and skeletal tissues, but yet, they have raised a number of questions. How related are different post-natal populations of post-natal stem cells and where do they reside within the tissue of origin? The protein compositions of bone, dentin, cementum and associated tissues are very similar, yet the organization of the extracellular matrix produced upon *in vivo* transplantation by bone marrow stromal cells, dental pulp cells and PDL cells are quite distinctive. Early attempts to identify differences in the transcriptomes between these different populations of post-natal SSCs were unremarkable, but with the evolution of new platforms in both genomics and proteomics, it is hoped that factors that specify the formation of one type of tissue versus another will be identified. Immunophenotyping revealed that the cells are indeed quite similar, and are uniformly negative for hematopoietic markers, and variably positive for connective tissue, endothelial, and smooth muscle markers. Two markers emerged, Stro-1 (an epitope found to be expressed by all bone marrow stromal CFU-Fs), and smooth muscle actin, as being consistently expressed by these different populations. Upon immunolocalization, both were found to be expressed in pericytes, cells found in close association with endothelial cells. Given the fact that vasculature is a common feature of virtually all tissues, and based on other evidences, pericytes are now considered to be a primary candidate for local stem cells in a variety of different tissues (Bianco et al, 2001a).

More global questions relate to the "intrinsic" character of post-natal stem cells; that is, what are the genes that control self-renewal and regulate differentiation into predictable cell phenotypes? Furthermore, there are questions concerning the 'extrinsic' factors; that is, what is the character of a stem cell 'niche', the three-dimensional structure and soluble factors that govern a stem cell's quiescence or activation, as required by the organism? To answer these questions, concerted efforts on all types of stem cells (embryonic, fetal, post-natal) are needed. Determining what controls unlimited replication and pluri-potentiality in embryonic stem cells may be applicable to post-natal stem cells in order to generate large numbers of autologous cells for regeneration of a numerous tissues without the fear of rejection. *Vice versa*, determining the pathways by which post-natal stem cells are so predictable in regenerating specific tissues will be required in order to harness embryonic stem cells and prevent their uncontrolled differentiation (teratoma formation).

APPROACHES AND DIMENSIONS OF TISSUE ENGINEERING

There are a number of ways in which it can be envisioned that tissues destroyed by disease or trauma can be regenerated, including: 1) encouragement of local stem cells via exogenous growth factors with or without scaffolds, 2) delivery of exogenous cells with or without growth factors, with or without carriers, and 3) construction of tissues and organs or their primordia, *ex vivo*. In all cases, it is now apparent that long-term efficacy is highly dependent on maintaining the activity of the pertinent stem cell. Furthermore, the dimensions of the tissue under reconstruction must be considered. Bone marrow transplantation is an example (and perhaps the only one) of tissue engineering in one dimension, whereby one fluid is replaced by another, and due to its simplicity, it is the most routine demonstration of

stem cell-based therapy to date. However, there have been recent advances in two-dimensional reconstruction in skin and corneal surfaces, based on development of techniques to maintain the epidermal stem cell within the populations used and identification of appropriate scaffolds that support their activity. The reconstruction of three-dimensional structures such as bones and teeth imposes a more difficult task in terms of maintaining the appropriate spatial relationships between different cell types, and importantly, in establishing appropriate vascularization (Bianco et al, 2001b; Robey et al, 2004).

EX VIVO EXPANSION, SCAFFOLDING, ANIMAL MODELS AND ASSESSMENTS

Establishment of optimal conditions of *ex vivo* expansion is the first step in developing a stem cell based tissue engineering strategy. Although it is often stated in the literature that 'stem cells' were established in culture, current culture conditions do not select for stem cells, *per se*, but rather support a subset of cells within the population that are true stem cells, based on *in vivo* transplantation assays. To date, there is no way to isolate a post-natal SSC separately from more committed cells (as indicated by clonal analysis) due to the lack of a specific marker, or to grow a pure population of stem cells. In fact, the latter may not be possible, based on current thinking. It is thought that stem cells undergo self-renewal via asymmetric division, whereby one daughter cell remains a stem cell and the other daughter becomes a more committed, transiently amplifying progenitor (dividing symmetrically), which ultimately gives rise to a non-proliferating, mature progeny. Consequently, even if stem cells could be isolated separately, asymmetric division would result in the stem cells becoming more diluted with time. However, regardless of the fact that the cell populations contain a decreased percentage of stem cells over time, the benefit of transplantation of more committed transiently amplifying cells to regenerate tissues that have a slow rate of turnover, such as bone and dentin, cannot be disregarded.

Current *ex vivo* culture conditions, including maintaining cells in a serum-free condition for a period of time, have been shown to support the formation of bone and dental structures when transplanted in association with an appropriate scaffold. While a number of different carriers have been developed, to date, HA/TCP (65%/35%) appears to be the most efficacious in support of bone, dentin, cementum and PDL like structures. However, HA/TCP is only slowly resorbed, and it would be highly beneficial to identify other scaffolds that would be equally as conductive, provide support, but be resorbed at a faster rate. Collagen sponges and other synthetic polymers have also been used with variable results, but show promise (Robey et al, 2004).

Using the current *ex vivo* expansion conditions and available scaffolds, a number of animal models have been developed to use *ex vivo* expanded SSCs from bone marrow to regenerate critical size defects (that will never heal on their own) in the craniofacial region (Fig 1). First in mice, and more recently in dogs, bone marrow stromal cells were isolated from femoral marrow, expanded *ex vivo*, and transplanted either with collagen sponges (in mice) or with HA/TCP (in dogs). In the mouse study, the defect completely filled in with bone of donor origin within four weeks as determined by histological methods (Krebsbach et al, 1998). In the dog study, software with a specialized algorithm and a clinical CT scanner were used to non-invasively determine new bone formation, even in the presence of the HA/TCP scaffold, something that cannot be done with standard plain radiographs. Again, defects filled with *ex vivo*-expanded cells and HA/TCP exhibited complete regeneration, verified at euthanasia by histological means. The major landmark of this study was not only the regeneration of bone in a large animal, but also the development of a non-invasive procedure for measurement of new bone formation, a necessary step in moving these pre-clinical studies towards human application (Mankani et al, 2004; Mankani and Robey, unpublished).

These results indicate the efficacy of direct orthotopic transplantation of SSCs along with a scaffold. However, there are instances in which the recipient site is hostile, due to extensive trauma and loss of local vascularity, infection, and effects of radiation. In another application designed to overcome donor site morbidity, vascularized bone grafts were prepared by incorporating *ex vivo* expanded (murine) bone marrow stromal cells into collagen sponges, placing these sponges around an artery and vein, and further surrounding the sponge with a non-porous membrane to prevent collateral vascularization. These constructs developed into newly formed bone/marrow rudiments that were completely perfused by the artery and vein that they surrounded, and could be lifted, intact, and placed in another site (Mankani et al, 2001). In fact, it is a variation of this procedure that has been recently used to generate a vascularized bone graft to reconstruct the lower jaw of a patient, lost due to extirpative surgery. A customized titanium cage was constructed to match the lower jaw of the patient, filled with bone marrow harvested from the iliac crest, and placed within a vascularized bed in the muscle of the back of the patient. Later, the graft was moved with vasculature intact, into the lower jaw and shown to be metabolically active (Warnke et al, 2004). This procedure may very well be hastened by using *ex vivo* expanded cell populations. However, recent studies indicate that SSCs isolated from

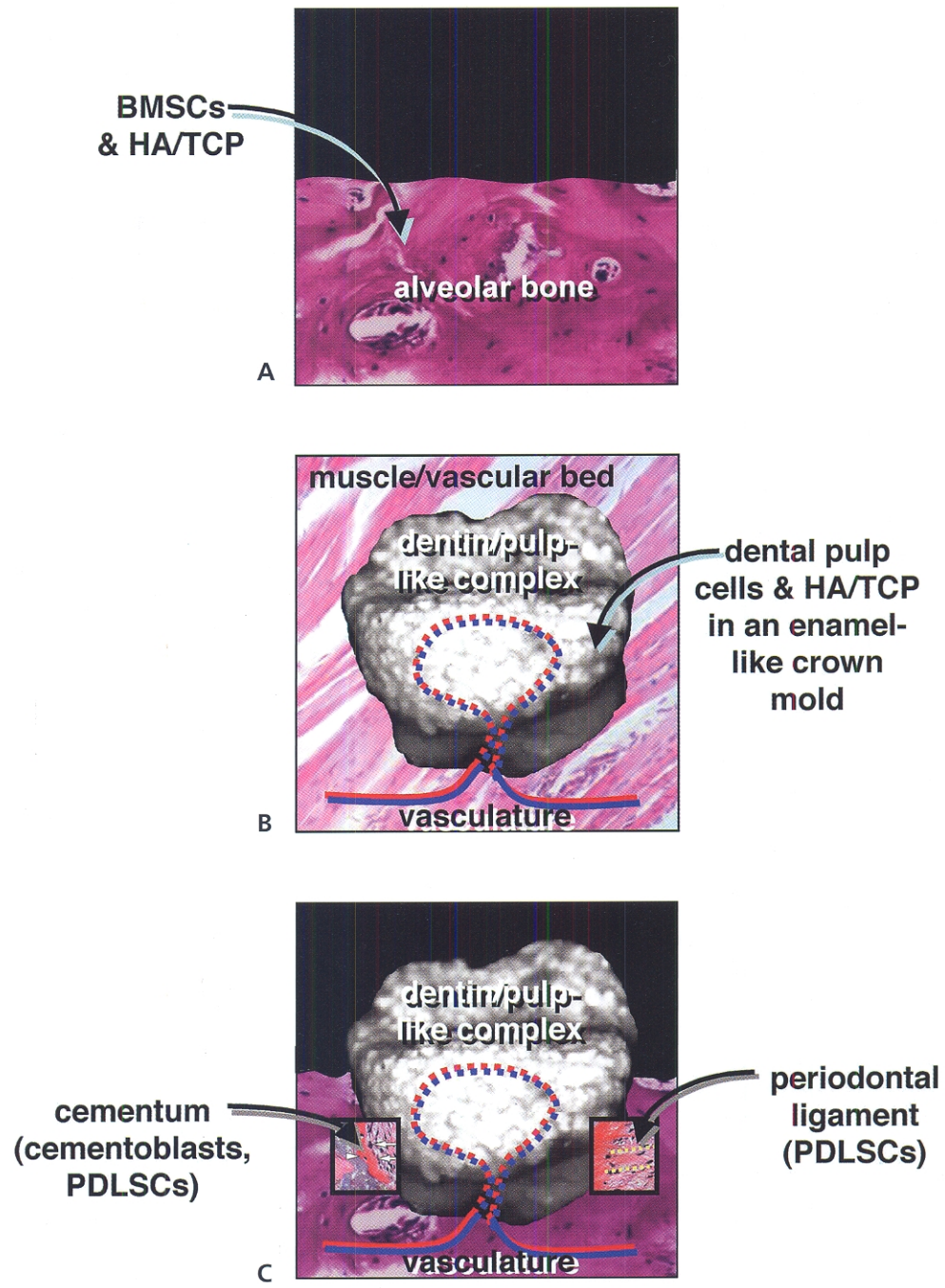

Fig 3 To build a tooth. **(a)** Teeth require bone for anchorage. In many cases, alveolar bone is lost due to periodontal disease and edentulous, and can be restored by transplantation of bone marrow stromal cells (BMSCs) with HA/TCP. **(b)** Meanwhile, a tooth could be constructed by placing dental pulp cells attached to HA/TCP within a crown mold composed of an enamel-like substance. This construct could be placed within a vascular bed in a muscle and allowed to consolidate. **(c)** Subsequently, the tooth structure with intact vasculature could be relocated to the jawbones, in conjunction with cells from the PDL, in order to recreate cementum and the PDL, which are essential components for normal function of teeth.

the craniofacial region, which derive from neuroectoderm in some instances, may display different characteristics compared to those isolated from the axial/appendicular skeleton, which derive from mesoderm (Akintoye and Robey, unpublished). Further investigation is required to determine the compatibility of skeletal stem cells derived from one site, and transplanted into another.

HOW TO BUILD A VIABLE TOOTH AND ASSOCIATED STRUCTURES?

While 'dentists' in ancient civilizations, such as the Etruscans, working approximately in the 700s B.C. developed elaborate systems to replace lost teeth, for the most part the loss of a tooth was considered to be an inevitable event by most populations around the world. In the 1700s and 1800s, concerted efforts were made to develop full dentures out of materials that could withstand the hostile environment created by the oral cavity. The subsequent development of dentures with enduring substances marked a major advance in restoring masticatory function to edentulous patients. However, it was realized that without the influences of normal masticatory mechanical forces, alveolar bone is quickly lost, as is the ability to support the use of dentures. The development of dental implants followed, as a way to restore a tooth (or teeth), in order to avoid the use of dentures - another major step forward, but still not without problems. The long-term efficacy of dental implants is not clear to date, due to the lack of appropriate interfaces between metal substances and bone. So what is the future?

The long-term goal is to develop a viable tooth. There are several ways that this could be accomplished. Recent studies indicate that cells from tooth buds can reorganize to form 'mini teeth' (Duailibi et al, 2004). Other studies indicate that oral epithelium in combination with appropriate mesodermal derivatives can also form tooth rudiments (Modino et al, 2005). The thought behind these studies is to develop a rudimentary tooth germ, that when transplanted into alveolar bone would undergo normal developmental processes and erupt to form a normal tooth. This is the ultimate goal of all efforts to replace a lost tooth. But an interim solution can be envisioned (Fig 3).

First, it is clear, one cannot have teeth without bone. Adequate alveolar bone is an absolute requirement for restoration of normal dentition and masticatory function. It is now possible to build new bone in the alveolar ridge using bone marrow-derived SSCs (Fig 3a). Next, based on technology used to develop vascularized bone grafts, it is conceivable that a viable tooth could be constructed by using a crown mold, composed of an enamel-like material, filled with dental pulp stem cells and HA/TCP, with a vascular bed inserted within the construct. This construct could be grown in a number of muscular sites (Fig 3b), and then transferred to the oral cavity. Subsequently, cells isolated from the PDL (isolated from the same individual's molar extraction) could be utilized to reform cementum and PDL, structures that are essential for normal function (Fig 3c). Although it is not yet clear if cementum is a distinct entity, or a variation of bone, its covering of the root surface appears to be essential for the attachment of the PDL and the formation of Sharpey's fibers. It is imperative that the function of the PDL in acting as a shock absorber during mastication be restored, no matter what the approach in regenerating a viable tooth.

SUMMARY

There have been a number of remarkable advances in the isolation and characterization of post-natal stem cells that form hard tissues, and the soft tissues associated with them that support their function. While much is yet to be learned about how these post-natal stem cells function, and what they ultimately can form, opportunities are emerging that indicate that the time is ripe to begin to develop clinical applications, including the building of a viable tooth.

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